PROTEIN S, A NEW VITAMIN K-DEPENDENT PROTEIN FROM BOVINE PLASMA

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Received 22 March 1979

1. Introduction

Five zymogens of serine amidases that require vitamin K for their biosynthesis have been purified from bovine plasma and chemically characterized. Prothrombin (factor II), factor VII, factor IX and factor X are the four traditional vitamin K-dependent clotting factors [1], whereas the fifth protein, called protein C [2] was isolated and characterized only recently [3–6]. Evidence has been presented [7] indicating that protein C has a regulatory function in blood coagulation. Protein C can be activated to a serine amidase by the factor X activator from Russel's viper venom or by thrombin [3]. All these proteins contain the vitamin K-dependent γ-carboxyglutamic acid residues. Another protein called protein S which occurs in very low concentration in human plasma was purified [8].

In the course of our purification of protein C from large volumes of bovine plasma we have found a previously unknown vitamin K-dependent protein, and devised a simple method for its purification. A monospecific antiserum against this protein did not react with any of the other vitamin K-dependent plasma proteins. The amino terminal sequence of this protein is similar to that of human protein S. Our antiserum against this protein also reacts with a vitamin K-dependent protein purified [9] and considered to be the bovine counterpart to human protein S. Our procedure for the purification of bovine protein S and some of its properties is described here.

2. Materials and methods

The vitamin K-dependent proteins were purified from slaughterhouse blood collected as in [2]. About 100 l blood were used in a typical preparation. The plasma was separated from the blood cells with a De Laval blood separator. The initial steps in the purification of protein S (barium citrate adsorption, ammonium sulphate fractionation and DEAE–Sephadex G-50 chromatography) were performed as in [2] except that the ammonium sulphate fractionation was at 20–67% saturation instead of 40–67%. The pooled fractions from the DEAE–Sephadex chromatography (fig.1) were dialyzed against 50 mM Tris–HCl, 0.1 M NaCl (pH 7.4) and applied to a column (2.5 × 40 cm) of blue dextran–Sepharose in the same buffer. The flow rate was 24 ml/h and 8 ml fractions were collected. Protein S was not retained on the column and appeared in the first protein peak whereas prothrombin was eluted with 1.0 M NaCl. In some experiments traces of factor IX were removed by chromatography on heparin–Sepharose as in [10]. The purified protein S was either stored frozen or dialyzed against distilled water and lyophilized.

SDS–acrylamide disc-gel electrophoresis was as in [11]. Agarose gel electrophoresis and crossed immunoelectrophoresis were performed using standard procedures [12,13]. Double immunodiffusion was as in [14]. Protein S was quantitated by electroimmunoassay [15] using a monospecific antiserum prepared as in [2]. The antisera against bovine factors IX, X, prothrombin and protein C were the same as in [2] whereas the antiserum against bovine factor VII was a gift from Dr E. Davie.

The amino acid composition of protein S was determined in acid hydrolysates (24, 48 and 72 h in 6 M HCl at 110°C in vacuo) with standard procedures [16] using a single column program on a Kontron amino acid analyser with a Durrum (DC4A) resin.

γ-Carboxyglutamic acid was determined in base hydrolysates as in [17] with synthetic γ-carboxyglutamic acid as a standard.

The amino terminal sequence of protein S was determined by the technique in [18] using a Beckman model 890 C sequencer. Two programs were used, a modification of the Beckman 1M Quadrol program which allows simple identification of γ-carboxyglutamic acid (P. Fernlund, J. S., in preparation) and a standard program for protein sequencing [19]. The phenylthiohydantoin residues were identified by high pressure liquid chromatography and thin-layer chromatography as in [6].

3. Results and discussion

During purification of protein C a previously unrecognized protein, later identified as protein S, was found at the front of the prothrombin peak during DEAE–Sephadex chromatography (fig.1). Prothrombin was removed by blue dextran–Sepharose chromatography. Some preparations were contaminated with factor IX which was removed by heparin–agarose chromatography [10].

A typical preparation from 100 l bovine blood gave 140 mg of protein S. A monospecific antiserum against protein S was used for quantitation of the

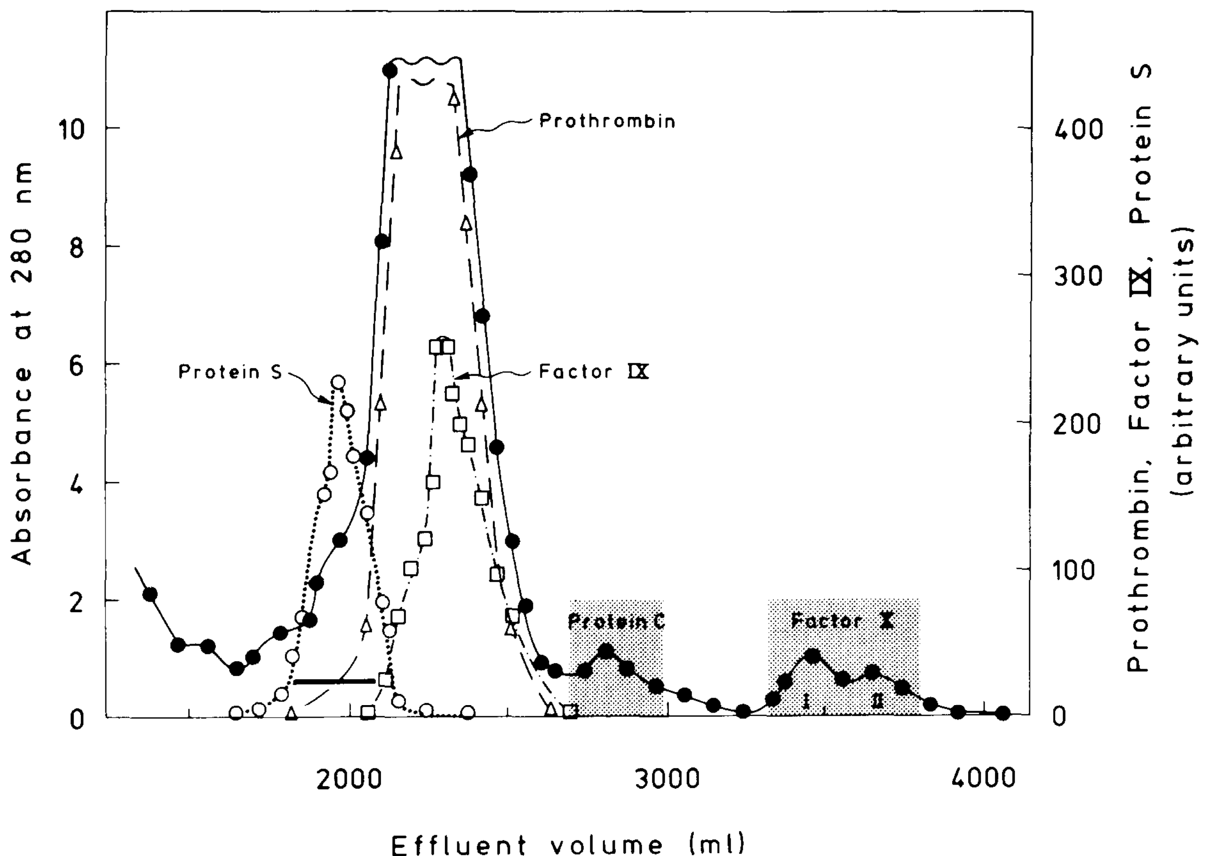

Fig.1. Chromatography of ammonium sulphate-fractionated material on a column of DEAE–Sephadex A 50 (5 × 52 cm) in 0.1 M phosphate buffer 1 mM benzamidine–HCl (pH 6.0). Elution was accomplished with a linear gradient of NaCl (0.15–0.55 M) 2000 ml in each chamber. The column was eluted at 90 ml/h and 10 fractions were collected per hour. The column effluent was monitored immunochemically with antisera against protein S, prothrombin and factor IX. The positions of the peaks containing protein C and factors X_1 and X_2 (shaded) were known from previous experiments.

protein by electroimmunoassay. The method did not allow precise measurements of protein S in plasma due to the low concentration of the protein. Barium citrate adsorption of the plasma however appeared to remove the protein quantitatively. The recovery of protein S measured from the barium citrate eluate was 35%.

On SDS–polyacrylamide gel electrophoresis purified protein S, both reduced and unreduced, appeared as a doublet (fig.2). This was not due to contamination with any of the previously characterized K-dependent plasma proteins since purified protein S did not react with antisera against any of these (fig.3). Protein S binds Ca^{2+} and its anodal electrophoretic mobility in agarose gel was lower in barbital buffer containing Ca^{2+} than in the same buffer containing EDTA instead of Ca^{2+} (fig.4). A single band was seen in both buffers.

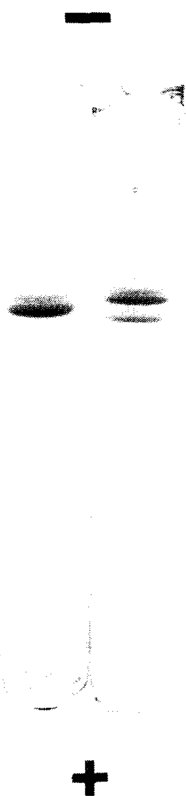

Fig.2. SDS–polyacrylamide gel electrophoresis of purified bovine protein S. Unreduced sample to the left and reduced to the right. About 7 μg protein was loaded on the gels.

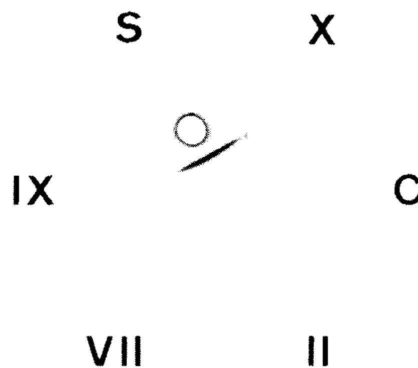

Fig.3. Double immunodiffusion of purified bovine protein S (center well) against antisera prepared against bovine factor VII, factor IX, factor X, prothrombin (factor II), protein C and protein S.

There was no heterogeneity in the amino terminal part of the molecule since sequenator degradation gave a unique amino terminal sequence and indicated that the protein was ≥ 90% pure. The reason for the heterogeneous appearance of the purified protein on SDS–acrylamide gels is not known.

SDS–acrylamide gel electrophoresis revealed that protein S consisted of a single polypeptide chain with an app. mol. wt 65 000 for the reduced protein based on the major protein band in the doublet. The amino acid composition of bovine protein S is shown in table 1. It was arbitrarily calculated relative to 30 residues of alanine which gives the apoprotein mol. wt

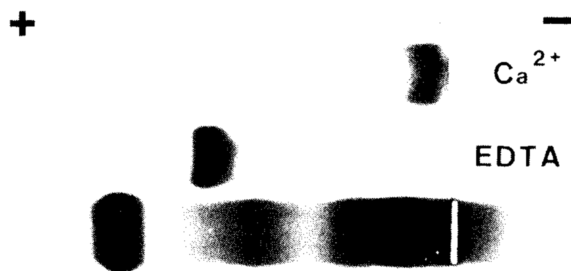

Fig.4. Agarose gel electrophoresis of purified bovine protein S in 0.075 M barbital buffer (pH 8.6) with 2 mM Ca^{2+} or 2 mM EDTA. The electrophoretic pattern of bovine plasma is shown as a reference.

Table 1
Amino acid composition of acid hydrolysate of bovine protein S[a]

Amino acid	Residues/molecule
Lysine	35.0
Histidine	7.2
Arginine	17.2
Half-cystine	26.0
Aspartic acid	58.1
Threonine[b]	27.5
Serine[b]	41.7
Glutamic acid	64.0
Proline	24.9
Glycine	37.9
Alanine	30
Valine[c]	39.0
Methionine	9.4
Isoleucine[c]	25.9
Leucine	41.7
Tyrosine	15.8
Phenylalanine	20.6
Tryptophan[d]	5.3
Total	527.2

[a] Calculated relative to thirty residues of alanine which gives a mol. wt 57 100 for the apoprotein
[b] Extrapolated to zero time hydrolysis
[c] 72 h hydrolysis value
[d] Determined spectrophotometrically [20]

57 100. Protein S contains hexose amine but it was not quantitated. The amino acid composition is in good agreement with that obtained [9]. The γ-carboxyglutamic acid content of protein S was 9.3 residues/mol protein when calculated relative to aspartic acid in the base hydrolysate. This can be compared with the γ-carboxyglutamic acid content of 10 residues/mol in prothrombin [17,21] and the 12 residues in factor X [22].

The amino-terminal sequence of bovine protein S was:
Ala–Asn–Thr–Leu–Leu–Gla–Gla–Thr–Lys–Lys–Gly–Asn–Leu
where Gla stands for γ-carboxyglutamic acid. Only 13 residues could be identified using either the modified program (P. Fernlund, J. S., in preparation) which allowed unambigous identification of γ-carboxyglutamic acid in positions 6 and 7 or a standard program for sequenator degradation of proteins. The sequence of bovine protein S shows a striking homology to the sequences of the other vitamin K-dependent plasma proteins as shown in fig.5.

The results presented here agree well with those in [9]. Our purification procedure is however shorter and does not expose the protein to denaturing conditions. γ-Carboxyglutamic acid was also positively identified during sequenator degradation. The function

Protein S	Ala	Asn	Thr	-	Leu	Leu	Gla	Gla	-	Thr	Lys	Lys	Gly	Asn	Leu
Prothrombin	Ala	Asn	Lys	Gly	Phe	Leu	Gla	Gla	-	Val	Arg	Lys	Gly	Asn	Leu
Factor IX	Tyr	Asn	Ser	Gly	Lys	Leu	Gla	Gla	Phe	Val	Arg	-	Gly	Asn	Leu
Factor X	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Val	Lys	Gln	Gly	Asn	Leu
Protein C	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Leu	Arg	Pro	Gly	Asn	Val
Factor VII	Ala	Asn	-	Gly	Phe	Leu	Gla	Gla	-	Leu	Leu	Pro	Gly	Ser	Leu

Fig.5. Comparison of the amino terminal sequences of bovine vitamin K-dependent plasma proteins. Data from [6,17,21–25]. The presence of γ-carboxyglutamic acid (Gla) in positions 6 and 7 in factor VII was inferred from the homology with the other proteins.

of protein S is unknown. Its similarity to the vitamin K-dependent clotting factors suggests that even this protein may be involved in blood coagulation.

Acknowledgements

The authors wish to express their sincere thanks to Dr Rickard DiScipio and Dr Earl W. Davie for making their manuscripts on protein S available prior to publication and for testing our antiserum against their protein S preparations. This investigation was supported by a grant from the Swedish Medical Research Council (B79-13X-04487-05C). We also thank Dr Ragnar Lundén of AB Kabi, Stockholm for making the De Laval blood separator available.

References

[1] Davie, E. W., and Fujikawa, K. (1975) Ann. Rev. Biochem. 44, 799–829.
[2] Stenflo, J. (1976) J. Biol. Chem. 251, 355–363.
[3] Kisiel, W., Ericsson, L. H. and Davie, E. W. (1976) Biochemistry 15, 4893–4900.
[4] Esmon, C. T., Stenflo, J., Suttie, J. W. and Jackson, C. M. (1976) J. Biol. Chem. 251, 3052–3056.
[5] Seegers, W. H., Novoa, E., Henry, R. L. and Hassouna, H. I. (1976) Thromb. Res. 8, 543–552.
[6] Fernlund, P., Stenflo, J. and Tufvesson, A. (1978) Proc. Natl. Acad. Sci. USA 75, 5889–5892.
[7] Kisiel, W., Canfield, W. M., Ericsson, L. H. and Davie, E. W. (1977) Biochemistry 16, 5824–5831.
[8] DiScipio, R. G., Hermodson, M. A., Yates, S. G. and Davie, E. W. (1977) Biochemistry 16, 698–706.
[9] DiScipio, R. G. and Davie, E. W. (1979) Biochemistry, in press.
[10] Fujikawa, K., Thompsen, A. R., Legaz, M. E., Meyer, R. G. and Davie, E. W. (1973) Biochemistry 12, 4938–4945.
[11] Laemmli, V. K. (1970) Nature 227, 680–685.
[12] Johansson, B. G. (1972) Scand. J. Clin. Lab. Invest. 29 (suppl. 124), 7–19.
[13] Ganrot, P. O. (1972) Scand. J. Clin. Lab. Invest. 29 (suppl. 124), 39–47.
[14] Ouchterlony, Ö. (1958) Progr. Allergy 5, 1–78.
[15] Laurell, C.-B. (1966) Anal. Biochem. 15, 45–52.
[16] Spackman, D. H., Stein, W. H. and Moore, S. (1958) Anal. Chem. 30, 1190–1206.
[17] Fernlund, P., Stenflo, J., Roepstorff, P. and Thomsen, J. (1975) J. Biol. Chem. 250, 6125–6133.
[18] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91.
[19] Thomsen, J., Bucher, D., Brunfeldt, K., Nexö, E. and Olesen, H. (1976) Eur. J. Biochem. 69, 87–96.
[20] Edelhoch, H. (1967) Biochemistry 6, 1948–1954.
[21] Morris, H. R., Dell, A., Petersen, T. E., Sottrup-Jensen, L. and Magnusson, S. (1976) Biochem. J. 153, 663–679.
[22] Thögersen, H. C., Petersen, T. E., Sottrup-Jensen, L. S., Magnusson, S. and Morris, H. R. (1978) Biochem. J. 175, 613–627.
[23] Fujikawa, K., Coen, M. H., Enfield, D. L., Titani, K., Ericsson, L. H. and Davie, E. W. Proc. Natl. Acad. Sci. USA 71, 427–430.
[24] Kisiel, W. and Davie, E. W. (1975) Biochemistry 14, 4928–4934.
[25] Bucher, D., Nebelin, E., Thomsen, J. and Stenflo, J. (1976) FEBS Lett. 68, 293–296.